In Vitro and Ex Vivo Assays to Assess Chemicals for Thyroid Hormone Synthesis Inhibition

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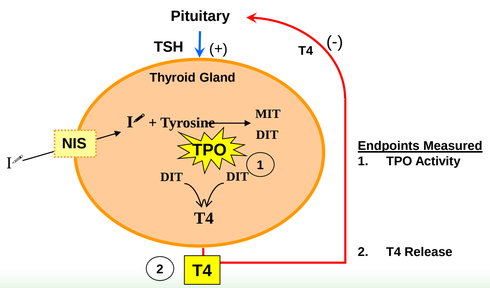
ABSTRACT

Disruption of thyroid hormone (TH) function may occur via multiple pathways including altered TH receptor binding, altered TH metabolism and elimination, and altered TH synthesis. To assess chemicals for their ability to affect TH synthesis, an *in vitro* enzyme inhibition assay and an *ex vivo* thyroid gland explant culture assay are being used. Thyroid peroxidase (TPO), the enzyme that catalyzes the iodination and coupling of tyrosines to produce TH, can be readily measured *in vitro*. This assay was used to determine the inhibitory potency of two model chemicals, methimazole and propylthiouracil (PTU), and a series of other chemicals of unknown thyroid disrupting activity. In the TPO inhibition assay, methimazole was the most potent of the two model inhibitors with an IC50 of 1.3 µM, whereas the IC50 for PTU was 11 µM. The other chemicals tested included a series of seven alkylphenols, two phthalates, three triazines, a thiazolone, triazole, thiazole, pyrazole, and imidazole, among others. None of the alkylphenols inhibited TPO when tested at concentrations to 3.6 mM. Two chemicals that inhibited TPO activity in vitro were further tested in a *Xenopus laevis* thyroid gland explant culture assay in which inhibition of T4 release was the measured endpoint. Dimethyl-hydroxymethylpyrazole inhibited T4 release but only at concentrations that were overtly toxic to the thyroid gland as measured by the MTT cytotoxicity assay. 2-Mercaptobenzothiazole inhibited T4 release from thyroid glands at non-cytotoxic concentrations and was more potent than methimazole for inhibition of T4 release. The action of this chemical on thyroid hormone function will be evaluated further in vivo in an *X. laevis*-based amphibian metamorphosis assay. This suite of assays provides a framework for determination of the potential for chemicals to affect thyroid hormone status.

OBJECTIVES

- Develop a rapid assay for assessing inhibitory effect of chemicals on thyroid hormone production
- Define inhibition dose responses for model inhibitors and estimate potency
- Test chemicals within defined chemical classes for their potential to inhibit thyroid hormone synthesis and to use this information to develop predictive models for thyroid hormone inhibition

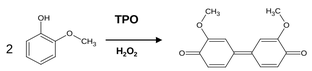


METHODS

1. Thyroid Peroxidase Inhibition Assay

The TPO inhibition assay is based upon guaiacol oxidation. This reaction is a surrogate for the TPO catalyzed coupling of iodo-tyrosines to form T4

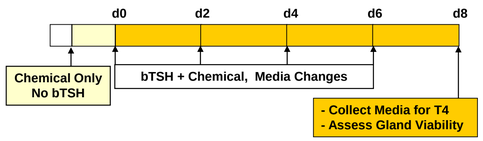
Guaiacol Oxidation[1]



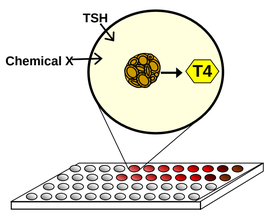
- Microsomes were prepared from porcine thyroid glands
- Microsomal protein (~ 150-200 µg total protein), guaiacol (35mM), and chemical were added to 96-well plate
- H_2O_2 (300 µM) was added to initiate the reaction and absorbance at 470 nm measured at 60s
- A full dose-response curve for methimazole was generated in parallel with each chemical as a positive control and to determine relative potency of test chemical to methimazole
- Solubility of chemicals in the reaction matrix was determined indirectly at the conclusion of the experiment by nephelometry (light scatter)

2. Thyroid Gland Explant Cultures

- Dissect thyroid glands from pro-metamorphic tadpoles
- Culture individually in 96-well plates in L-15 media with 0.1% BSA, antibiotic, and 1 µM KI ; 21°C
- Stimulate with bovine TSH alone (positive control) or TSH and graded concentrations of test chemical for 8 d



- Measure T4 released to media from d6 to d8 by RIA
- Gland viability is determined by MTT assay.



RESULTS

1. Inhibition of Thyroid Peroxidase Activity

Model Thyroid Synthesis Inhibitors

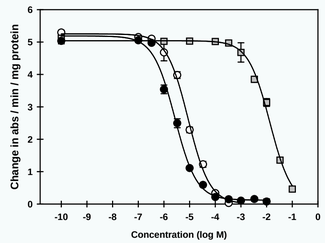


Figure 1. Methimazole is the most potent of the model T4 synthesis inhibitors in the TPO inhibition assay. Perchlorate had very low potency for TPO inhibition, but was the most potent of the three inhibitors in the *ex vivo* and *in vivo*[4,5] assays.

	IC50 (µM)
Methimazole	2.7
Propylthiouracil	8.8
Perchlorate	13,400

Test Chemicals

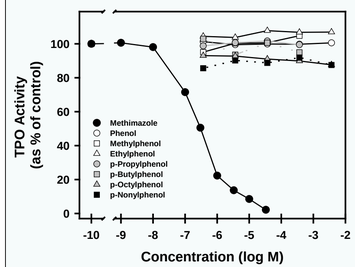


Figure 2. Alkylphenols did not inhibit TPO.

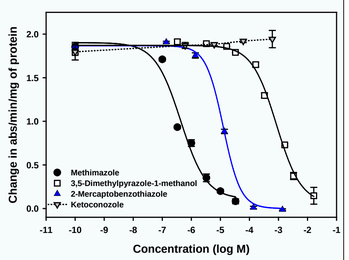


Figure 3. Two potentially positive chemicals identified.

Summary Table of Thyroid Peroxidase Inhibition Assays

Chemical Name	Chemical Class	Relative TPO Inhibition IC50 Methimazole IC50 "X"	IC50 (µM)	% Max Inhibition	n	toxicity in glands
Methimazole	imidazole	1	2	100	36	NO
Propylthiouracil	thiourea	0.17	6	100	4	n.t.
Perchlorate	small anion	0.00011	10321	100	5	n.t.
2-Mercaptobenzothiazole	thiazole	0.042	14	100	4	NO
Sodium dichloroisocyanurate	triazine	0.0064	242	70	6	y ?
3,5-Dimethylpyrazole-1-methanol	pyrazole	0.0046	946	100	3	yes
Iopanoic acid	iodo phenyl	0.0021	595	100	4	n.t.
2-(2-Hydroxy-5-methylphenyl)benzotriazole	triazole	No Inhibition	No Inhibition	0	2	n.t.
Ketoconazole	imidazole	No Inhibition	No Inhibition	0	2	n.t.
1,2-benzisothiazol-3-one	thiazolone	No Inhibition	No Inhibition	0	2	n.t.
N,N',N-trichloro-1,3,5-triazine-2,4,6-triamine	triazine	No Inhibition	No Inhibition	0	2	n.t.
Terbuthylazine	triazine	No Inhibition	No Inhibition	0	2	n.t.
Triclosan	OH-PCDE	No Inhibition	No Inhibition	0	2	n.t.
Phenol ... to ... nonylphenol	alkyl phenol	No Inhibition	No Inhibition	0	2 ea	n.t.
nonylphenol - (straight chain)	alkyl phenol	No Inhibition	No Inhibition	0	2	n.t.
di-ethylphthalate	phthalate	No Inhibition	No Inhibition	0	2	n.t.
benzylbutylphthalate	phthalate	No Inhibition	No Inhibition	0	2	n.t.
Amiodarone	mixed iodo phenyl	No Inhibition	No Inhibition	0	2	n.t.

2. Inhibition of T4 Release from Cultured Xenopus Thyroid Glands

Model Thyroid Synthesis Inhibitors

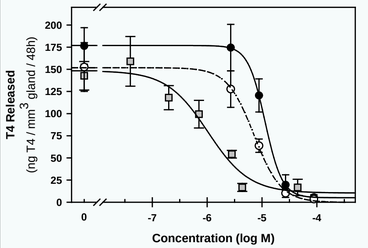


Figure 4. The potency of Methimazole and PTU for inhibiting TSH stimulated T4 release from thyroid gland explant cultures is similar to that for inhibiting TPO activity (Figure 1). Perchlorate IC50 of 1 µM is the same as the KI concentration in the culture media.

Chemical	IC50 (µM)
Perchlorate	1
Propylthiouracil	7
Methimazole	11

Chemicals Positive in TPO Assay tested in Thyroid Gland Explant Culture

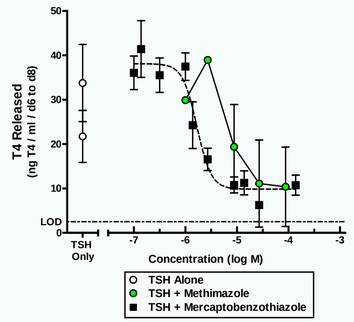


Figure 5. 2- Mercaptobenzothiazole(square symbols) inhibited T4 release from thyroid glands with slightly greater potency than methimazole (green circles), but was slightly less potent than methimazole when tested in the TPO assay (Figure 3).

The 3,5-dimethylpyrazole-1-methanol that was an apparent inhibitor of TPO activity (Figure 3) was tested in the thyroid gland explant assay but did not inhibit T4 release from explant cultures at concentrations below those that were toxic (≥ -3logM) as determined by MTT assay (data not shown).

SUMMARY

- The results of the TPO assay correspond well with the *ex vivo* assay system when TPO inhibition is the primary mechanism of action of the chemical. Differences in potency may indicate different, or multiple mechanisms of thyroid hormone synthesis inhibition
- Chemicals that show inhibitory activity in the TPO inhibition assay need to be tested further in the *ex vivo* thyroid gland culture assay to confirm their effects on thyroid hormone synthesis and release, and can be further verified in higher level thyroid toxicity assays such as the amphibian metamorphosis assay.
- This suite of assays can be an effective tool to determine the capacity of previously untested or unsuspected classes of chemicals to disrupt normal thyroid hormone production and to develop predictive models incorporating structure activity relationships between chemical structure and T4 synthesis inhibition

References:

[1] Doerge et al. 1997. *Anal. Biochem.* 250, 10-17.
[2] Divi and Doerge, 1996. *Chem. Res. Toxicol.*, 9, 16-23.
[3] Schmutzler et al. 2004. *Toxicology* 205, 95-102.
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[5] Tietge et al. 2005. *Environ. Toxicol.Chem* 24, 926-933.

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